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PRINCIPAL INVESTIGATOR: Lu-Hai Wang, Ph.D.

CONTRACTING ORGANIZATION: Mount Sinai School of Medicine
New York, NY 10029-6574

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14. ABSTRACT Twenty one human breast cancer lines were screened for expression of various RPTK including ErbB2, EGF, IGF-1 and insulin receptors. Those lines displayed a wide spectrum of colony forming and invasion activity. ErbB2 over expressing cells displayed an increased sensitivity toward inhibitors of the PI3 kinase signaling pathway including rapamycin for monolayer and anchorage independent growth. Forced expression of ErbB2 in the non-ErbB2 over-expressing cells was able to confer the rapamycin hypersensitivity of those cells for anchorage independent growth. Rapamycin and herceptin additively inhibited adherent and anchorage independent growth of ErbB2 over-expressing breast cancer cells. An ErbB2 transgenic mouse model and the nude mouse xenograft model were used to test the tumor inhibitory effect of rapamycin and herceptin. Our result showed that rapamycin and herceptin each displayed a significant inhibition, and together demonstrated an additive inhibition, of tumor growth and tumor incidence, and thus combination of the two drugs could offer an improved anti-breast cancer therapy.					
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Table of Contents

Cover.....	
SF 298.....	
Table of Contents.....	
Specific Aims.....	1
Background.....	2
Method.....	3
Results.....	5
Publications.....	16
Literature Cited.....	20

Final Report

Award No.: DAMD17-02-1-0504

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Title: Exploring the Molecular Targets for breast cancer therapy

P.I.: Lu-Hai Wang

Institute: Mount Sinai School of Medicine

A. Specific Aims:

1. To test if overexpression or activation of components of the PI3 kinase pathway results in the sensitization of non-ErbB2-overexpressing cancer cells to the effects of inhibitors of the PI3 kinase signaling pathway.

(i) Effect of expressing constitutively active (ca) PI3 kinase, Akt or p70S6 kinase on the sensitivity of breast cancer cells with low ErbB2 expression to inhibitors of PI3 kinase and p70S6 kinase.

(ii) Analysis of cell cycle progression and its regulatory check point proteins in the breast cancer lines with ErbB2 overexpression or activated PI3 kinase signaling molecules after treatment with the inhibitors of ErbB2 (herceptin), PI3 kinase (LY294002) or p70S6 kinase (rapamycin).

2. Further screening of ErbB2-overexpressing breast tumors for their sensitivity to herceptin, LY294002, and rapamycin.

(i) Assessment of additional established ErbB2-overexpressing breast cancer lines for sensitivity to PI3 kinase pathway inhibitors.

(ii) Testing primary ErbB2-overexpressing breast cancer tissue for their sensitivity to PI3 kinase inhibitors alone or in combination with herceptin by *in vitro* assay of short-term organ culture.

3. To test and compare the effect of PI3 kinase pathway inhibitors and in combination with herceptin on tumorigenicity and the *in vitro* and *in vivo* metastatic ability of breast cancer cells with and without ErbB2 overexpression.

(i) To test and compare the effect of the PI3 kinase pathway inhibitors and in combination with herceptin on the *in vitro* invasive ability of breast cancer cells with and without ErbB2 overexpression.

(ii) To test the effect of PI3 kinase pathway inhibitors and herceptin on the tumorigenicity of ErbB2-overexpressing versus non-ErbB2-overexpressing cancer cells in xenograft nude mice.

(iii) To compare the effect of PI3 kinase pathway inhibitors alone or in combination with herceptin on the development and progression of breast tumor in the ErbB2-mediated and non-ErbB2-mediated transgenic mouse mammary tumor models.

B. Background

ErbB2, a member of the epidermal growth factor receptor (EGFR) family, has been found to be overexpressed and/or amplified in about 30% of human breast cancers (1). Amplification of ErbB2 is associated with a high relapse rate and poor clinical prognosis (2-4). Both in-vitro and in-vivo studies have demonstrated the functional role of ErbB2 in cell transformation and mammary cell carcinogenesis. Overexpression of ErbB2 led to transformation of NIH3T3 cells and mammary epithelial cells (5-8). Down modulation of ErbB2 by a monoclonal antibody reverted the transformed phenotype of ErbB2 transformed NIH3T3 cells (9). Mammary tumor virus promoter-based c-ErbB2 transgenic mice developed mammary carcinoma (10, 11). Spontaneous ErbB2 amplification or ectopic overexpression of ErbB2 leads to activation of PI3 kinase signaling, which has been demonstrated to play an important role in ErbB2-mediated cell transformation and mammary malignancy, in part via regulating genes affecting cell cycle, growth and drug sensitivity (12-17). Activation phosphorylation of Akt, mTor and 4E-Bp1 was shown to correlate with ErbB2 overexpression, tumor progression and poor prognosis (17).

Thus the components of the ErbB2/PI3 kinase pathways represent rational targets for human breast cancer therapy. A humanized monoclonal antibody against ErbB2, trastuzumab or herceptin was developed (18) and used subsequently for therapy in patients with metastatic breast cancer involving overexpression of ErbB2 (19-22). Clinical trials so far indicate that as a first line single agent, herceptin can achieve an objective response of 15 to 30% and can significantly improve the response rate of tumor progression interval and median survival when combined with chemotherapy such as paclitaxel (19, 23, 24). However, the overall response rate with herceptin alone or in combination with chemotherapy underscores the need of an improved therapeutic regimen for breast cancer. We have shown previously that ErbB2-overexpressing human mammary carcinoma cells display an increased sensitivity toward inhibitors of the PI3 kinase signaling pathway, including rapamycin, for anchorage independent growth and that non-ErbB2-overexpressing cells could be sensitized for the rapamycin inhibition for growth by ectopic overexpression of ErbB2 (25). The inhibition was due to, at least in part, the decrease of cyclin-associated CDK activity (25).

Rapamycin forms complexes with immunophilin FK506-binding protein 12 leading to specific inhibition of the serine/threonine protein kinase mTOR or mammalian target of rapamycin (26). mTOR is a downstream component of the PI3 kinase signaling of which the two major phosphorylation targets are 40S ribosomal protein kinase, p70 S6 kinase, and a translation repressor 4E-BP1 (eIF4E-binding protein 1) (27-29). Phosphorylation of p70S6 kinase and 4E-BP1 results in their activation and inactivation respectively (27-29). Therefore, inhibition of the mTOR by rapamycin results in decreased translation of the RNAs involved in cell cycle progression and proliferation leading to growth arrest and apoptosis (30-32). Rapamycin and its analogs are being developed as therapeutic drugs by themselves or in combination with other chemotherapy drugs for various types of human malignancy including breast cancer (33-36). A recent study showed that rapamycin synergistically enhanced paclitaxel- and carboplatin- induced cytotoxicity in ErbB2-overexpressing breast cancer cells (37). However, to our knowledge there have not been reports on preclinical or clinical studies on the combination therapy for breast cancer using rapamycin and herceptin.

The purpose of this study is to confirm and extend our previous finding that ErbB2-overexpressing breast cancer cells display an increased requirement of PI3 kinase signaling for anchorage independent growth and to evaluate the anti-cancer effect of rapamycin by itself or in

combination with herceptin in both *in-vitro* and *in-vivo* systems. The results confirm our previous observation of hypersensitivity of ErbB2 over-expressing breast cancer cells toward rapamycin for anchorage independent growth. Moreover, rapamycin is able to enhance herceptin-mediated growth inhibition *in-vitro* and tumorigenicity *in-vivo* of ErbB2 overexpressing human breast cancer cells.

It is also the objective of this study to elucidate the molecular basis for cell invasion and tumor metastasis. For this purpose, we have developed an *in-vitro* protocol to isolate highly invasive cells from low or moderate invasive breast cancer cells. The selected cells were compared with their parental counterpart cells for biological properties including invasion activity and molecular signaling profile in an attempt to identify the molecular basis underlying the invasion activity.

C. Methods

Cell culture and reagents.

Some of the established normal human breast epithelial and breast cancer lines have been described previously (25). Additional lines used in this study have been obtained from ATCC. Cells were grown in DMEM containing 10% FBS. Some of the lines (T47D, Hs57T, MCF7, BT483, BT549, MDA-MB-330 and MDA-MB-415) were maintained in the same medium supplemented with insulin at 50 ng/ml. An ErbB2 overexpressing, highly tumorigenic subline called MCF7-I4 was derived from the original MCF7 line by the selection protocol described below.

Preparation of transient tumor organ culture, tumor-derived cell culture and drug sensitivity test

A portion of the freshly procured ovarian tumor specimen was subjected to short term organ culture. Nylon cloth with appropriate mesh was cut into 2.5 cm² pieces and coated with paraffin around the edge and stored in 75% ethanol. Prior to use, the nylon cloth was washed with sterile water and soaked in the growth medium. Appropriate volume of culturing medium was added. One mm sections of fresh tumor tissue were placed on top of the cloth platform floating on the surface of the medium. The cells were nourished by the nutrients diffusing through the mesh of the cloth. Medium was changed once every 3 to 4 days. Under such condition, ovarian tumor tissues could be maintained for 3 to 4 weeks with some degree of growth from cells around the outskirts of the specimen. ³H-Thymidine was used to label the mitotic cells in the presence or absence of the inhibitor rapamycin (5 - 10 ng/ml) alone or in combination with carboplatin.

To assay for other oncogenic properties and their sensitivity toward the drugs, short term tumor cell cultures were prepared from well characterized ovarian tumor specimens. We have recently adopted a culturing method using methyl cellulose medium to grow transformed fibroblasts and epithelial cells in an anchorage independent condition (38). Under such condition, normal fibroblasts will undergo growth arrest and epithelial cells will undergo apoptosis in 24 to 48 hours, whereas transformed cells will survive and proceed through the cell cycle although in a limited fashion (38). Taking advantage of this important property of transformed and cancer cells, namely resistance to anoikis, the tumor cell suspension was prepared from the fresh specimen using trypsin (0.5%) and collagenase (0.1%) and grown in methylcellulose medium for 24 to 48 hours to select against the contaminating normal and

inflammatory cells, the cells were harvested and seeded on petri dishes pre-coated with Matrigel. Twenty four hours later, the cells were harvested and subjected to further analysis including monolayer growth, and invasion assay with or without the inhibitors rapamycin and carboplatin individually or in combination.

Pharmacological inhibitors.

Tissue culture grade LY294002, PD98059 and rapamycin were purchased from LC laboratories. The humanized mouse monoclonal antibody against the human ErbB2, herceptin or trastuzumab, was provided by Genentech Co. via a material transfer agreement. Rabbit antiserum against the cytoplasmic domains of the insulin and IGF-1 receptors have been prepared and described previously (39,40). Antibodies against ErbB2 and EGF receptors were purchased from Calbiochem and Santa Cruz Biotechnology. Antibodies against AKT1 and AKT2 were also purchased from Santa Cruz Biotechnology. Antibodies against phospho-AKT, MAP kinase, phospho-MAP kinase, p70 S6 kinase and phosphor-p70 S6 kinase were purchased from Cell Signaling.

Assay of cell growth, migration, invasion and colony formation.

For testing the effect of drugs on the growth of cell in monolayer, 1×10^5 cells were seeded in a duplicate 6 cm dishes in regular maintenance medium. Twenty four hours later the cultures were replenished with fresh medium with or without the drug to be tested and the number of cells were counted 3 days later. The assay for cell migration has been described previously (41). The assay for cell invasion was similar to that of migration except that the cell culture inserts were coated with matrigel as follows: 0.1ml of DMEM containing 0.1% BSA and 0.5 μ g/ml of growth factor reduced matrigel was added to each insert and allowed to sit at room temperature for 15 minutes. The inserts were then incubated at 37°C for 0.5 – 2 hrs. 30 minutes before adding cells to the inserts 400 μ l of DMEM containing 0.1% BSA was added to hydrate the matrigel. The remainder of the assay proceeded as in the migration assay. Colony assays followed the procedures described previously (42-44).

In-vitro selection of highly invasive cells.

To select for invasive cells larger inserts and companion plates were used (Falcon #3093 and 3502) and the invasion assay scaled up. 2×10^6 cells in a volume of 1.5 ml were used with 2.5 ml of 10% FCS DMEM as the chemoattractant. After 24 hr of invasion assay, the invaded cells were recovered by trypsinization and amplified for the next cycle of selection. Four cycles of selection were performed for MCF7 cells to obtain the MCF7I4 line

Protein analysis.

Preparation of total cell lysates, determination of protein concentration, immunoprecipitation and western analysis followed the previously described procedures (44).

Tumorigenicity test.

For xenograft model, the MCF7 I4 line and female BALB/C athymic mice were used. 2×10^6 cells in 0.1 ml PBS were injected subcutaneously at the flank per mouse. One week later mice were injected at the site of tumor with 0.1 ml PBS or 0.1 ml PBS containing 100ng/ml of rapamycin, 100 μ g/ml herceptin or 100ng/ml of rapamycin and 100 μ g/ml herceptin. The body

weight and tumor size were measured twice a week. At thirty days post tumor cells injection, which was set as the endpoint, the mice were dissected and examined for possible metastasis. For transgenic mouse model, the FVB/N-TGN (CMMTVneu) mice from Jackson laboratories were used. The transgenic mice harbor the human ErbB2 under the control of the mouse mammary tumor virus LTR promoter (46). Eight week old mice were purchased. The drug injection was started when mice reached 4.5 months old. Four groups of mice were individually injected intraperitoneally twice a week with PBS, 100ng/ml of rapamycin in 0.1 ml PBS, 100µg/ml herceptin in 0.1 ml PBS or a combination of rapamycin and herceptin. Sixteen injections were performed. The body weight and appearance of tumors and tumor sizes were monitored twice a week. The endpoint was set when mice reached 9.5 months old and most of the control mice developed tumors.

D. Results

I. Rapamycin as a therapeutic modality for treatment of breast cancer especially those with ErbB2 overexpression

Expression of RPTKs, AKT and MAP kinase signaling molecules in various human breast cancer cell lines.

To further investigate the relationship between ErbB2 overexpression in breast cancer cells and their sensitivity toward the inhibitors of the PI3 kinase signaling pathway as we reported previously (25), various breast cancer cell lines were analyzed for the expression and activation of insulin receptor (IR), IGF-1 receptor (IGF-1R), epidermal growth factor receptor (EGFR) and ErbB2 as well as their down stream signaling molecules including AKT, p70S6 kinase and MAP kinase. The results showed that out of 19 lines examined about half of them displayed varying degrees of elevated ErbB2 expression. Several of them, including MDA-MB-468, BT20, MDA-MB-231, showed relatively high EGFR expression. All of them had significant levels of IR and IGF-1R. MCF7, T47D, BT20, MDA-MB-436 had particularly high levels of IGF-1R. There appeared not to be a correlation between expression levels of AKT, MAP kinase and p70S6 kinase and their state of activation reflected in the degree of phosphorylation. The lines displayed varying degrees of constitutive activation of AKT, MAP kinase and p70S6 kinase.

Anchorage independent growth, invasion activity and inhibition by inhibitors against the PI3 and MAP kinase signaling pathways.

The colony forming ability and invasion activity of the various breast cancer cell lines were examined. The results show that they exhibit differential abilities for anchorage independent growth and invasion activity (Fig. 2). However, there is no direct correlation between the ErbB2 expression level and the colony forming or invasion activity. Confirming our previous observation, the anchorage independent growth of ErbB2 overexpressing lines displayed a greater sensitivity than the non-ErbB2 overexpressing lines toward the inhibitors of the PI3 kinase including LY294002 and rapamycin (Fig. 3A). All of them, however, were relatively resistant to inhibition by PD98059. The invasion activity of the lines showed different degrees of sensitivity toward LY294003, PD98059 and rapamycin (Fig. 3B) as well as an AKT inhibitor (data not shown). However, it appears not to have a correlation between ErbB2 overexpression and the sensitivity of invasion activity toward inhibition by LY294002 or

rapamycin similar to that observed for anchorage independent growth. Overall, there was no direct and simple correlation between the potency of anchorage independent growth or invasion activity with the expression level or activation status of the RPTKs or their downstream signaling molecules examined for the over 20 breast cancer lines tested here.

Effect of forced expression of ErbB2 or ca Akt in non-ErbB2 over expressing breast cancer cells on their sensitivity toward inhibitors of the PI3 kinase signaling pathway.

We have shown previously that forced expression of ErbB2 in MCF7 and MB231 cells render them more sensitive to inhibition by LY294002 and rapamycin for colony formation (25). The effect of exogenous ErbB2 over expression on invasion activity and the sensitivity by inhibitors of the MAP kinase and PI3 kinase signaling pathways was similarly examined. There appears not to have a direct correlation between the level of ErbB2 expression and invasion activity among the MCF-7 or MB231 clones, and those clones display varying degree of sensitivity toward the inhibitors for invasion (Fig. 4A). To further explore the role of PI3 kinase signaling molecules, an activated Akt (caAkt) was introduced into the MCF7 and MB231 lines to establish caAkt over expressing lines (Fig. 4B). Those caAkt over expressing MCF7 and MB231 lines were analyzed for colony forming ability and migration/invasion activity with or without the drugs. The relative Akt level for the MCF7 Akt lines is Clone 4 = 13 > 19 = 20 = pool > 5 > 3 = 2 = C5; and that for MB231 Akt lines is clone 12 = 16 > 3 = 4 > 2 = 6 = C1 (Fig. 4B). Although some of the caAkt over expressing lines have a tendency of yielding larger colonies, they had no significance difference in drug sensitivity for colony forming ability comparing to the parental line (data not shown). The individual lines display different potency in invasion activity, and there is no correlation with the caAkt expression level (data not shown). Similarly, the profile of the sensitivity of invasion activity toward inhibition by LY294002, PD98059 and rapamycin shows no overt relationship with the caAkt expression (Fig. 4B). However, the MCF7 lines seem to be more resistant to the drug inhibition for invasion. Moreover, in many cases, rapamycin increased instead of inhibiting the invasion activity in both MCF-7 and MB231 clones. The basis for this phenomenon is not clear and it needs to be further investigated.

Inhibition of monolayer and anchorage independent growth of breast cancer cells by rapamycin and herceptin.

The inhibitory effect of rapamycin and herceptin individually or in combination on the growth of ErbB2 overexpressing and non ErbB2 overexpressing breast cancer cell lines was assessed. The results showed that the growth in monolayer of ErbB2 overexpressing lines, BT474, HCC1419 and HCC1954 was more sensitive than the non-ErbB2 overexpressing lines, Hs578T, MCF7 and MDA-MB-231, to the inhibition of rapamycin and herceptin individually or in combination (Fig. 5A). The non-ErbB2 overexpressing lines were relatively resistant to the herceptin inhibition. In each case of the ErbB2 overexpressing lines, combination of rapamycin and herceptin gave a greater inhibition than either drug alone (Fig. 5A). Although the cell count did not show a dramatic decrease for herceptin treated HCC1954 and HCC1419 cells, the morphology of the cells suggested that most of them were undergoing apoptosis at 72 hrs post treatment. The ErbB2 overexpressing lines BT474, HCC1419 and HCC1954 were further tested for inhibition on anchorage independent growth. The results showed that the colony forming activity of all three lines was inhibited by rapamycin or herceptin and a greater inhibition was observed with their combination (Fig. 5B). Although the inhibition of HCC1419 in terms of number of colonies was not as significant as that for BT474 and HCC1954 the size distribution

of the colonies showed that significant inhibition of colony size was observed with rapamycin and especially with the combination of rapamycin and herceptin. The effectiveness of herceptin treatment in inhibiting ErbB2-mediated activation of ERK2 is shown in Fig 5C.

Inhibition of rapamycin and herceptin on tumorigenicity.

In a xenograft model MCF7-I4 cells were inoculated into BALB/C athymic mice. Subsequently the mice were treated with control vehicle solution, rapamycin, herceptin or combination of rapamycin and herceptin. The results showed that rapamycin or herceptin alone significantly inhibited the growth of tumors. Combination of the two drugs dramatically reduced the growth of tumors (Fig. 6A). Next the FVB/N-TGN (CMMTVneu) mice harboring the unactivated ErbB2 gene under the control of the MMTV LTR promoter (46) were used to further assess the inhibition of tumorigenicity by rapamycin and herceptin. It was reported that 50% of the female transgenic mice developed tumor at 205 days of age (46). In our experiment, the earlier onset of overtly visible tumors was observed in control, rapamycin and herceptin groups at 180 days after birth. At 248 days after birth 50% of the control group, 40% of the herceptin treated group and 10% of the rapamycin plus herceptin treated group developed overtly visible tumors. The tumor growth was significantly inhibited by herceptin and rapamycin and especially by the combined treatment of the two (Fig. 6B). All mice except one in the combined treatment group were alive at the end point which was set at 260 days after birth. Two-thirds of the tumor bearing mice in the control group carried multiple tumors at the endpoint, whereas only one-third and one-fifth of the tumor bearing mice in the herceptin and rapamycin groups, respectively, carried multiple tumors (Table 1), none of the tumor bearing mice in the combined drug treated group carried multiple tumors. The average tumor weight per mouse of the rapamycin, herceptin and combined drug treatment group was 28%, 56% and 16% of that of the control group (Table 1). No detectable adverse toxicity effect on the treated mice was observed for all the treatment groups described above as monitored by body weight and activity of the mice. The result from the xenograft and transgenic models demonstrate that combination of herceptin and rapamycin has significantly enhanced anti-tumorigenicity over that of either drug alone.

In conclusion, our results suggest that rapamycin together with herceptin could offer an improved anti-breast cancer therapy, especially for those with ErbB2 over-expression.

Figures

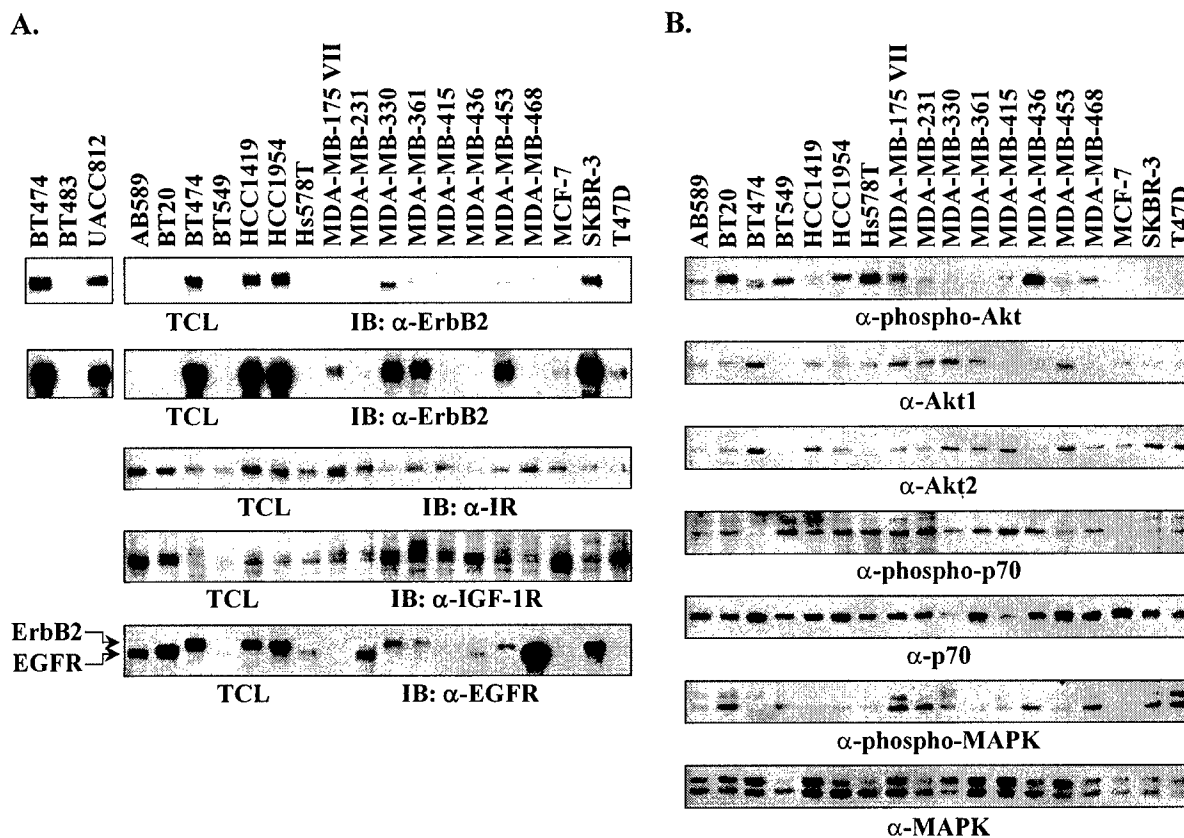


Fig. 1. RPTKs and signaling molecules in various breast cancer cell lines. Total cell lysates were prepared from various breast cancer cell lines and 20 μ g of total cell lysate was separated by SDS-PAGE followed by western blotting with either the indicated antibodies against ErbB2 and other growth factor receptors (A.) or antibodies against the indicated PI3 and MAP kinase signaling molecules (B.). Protein was detected via Western-STAR detection reagent (Perkin Elmer). A long exposure of the ErbB2 blot is shown (A. second panel).

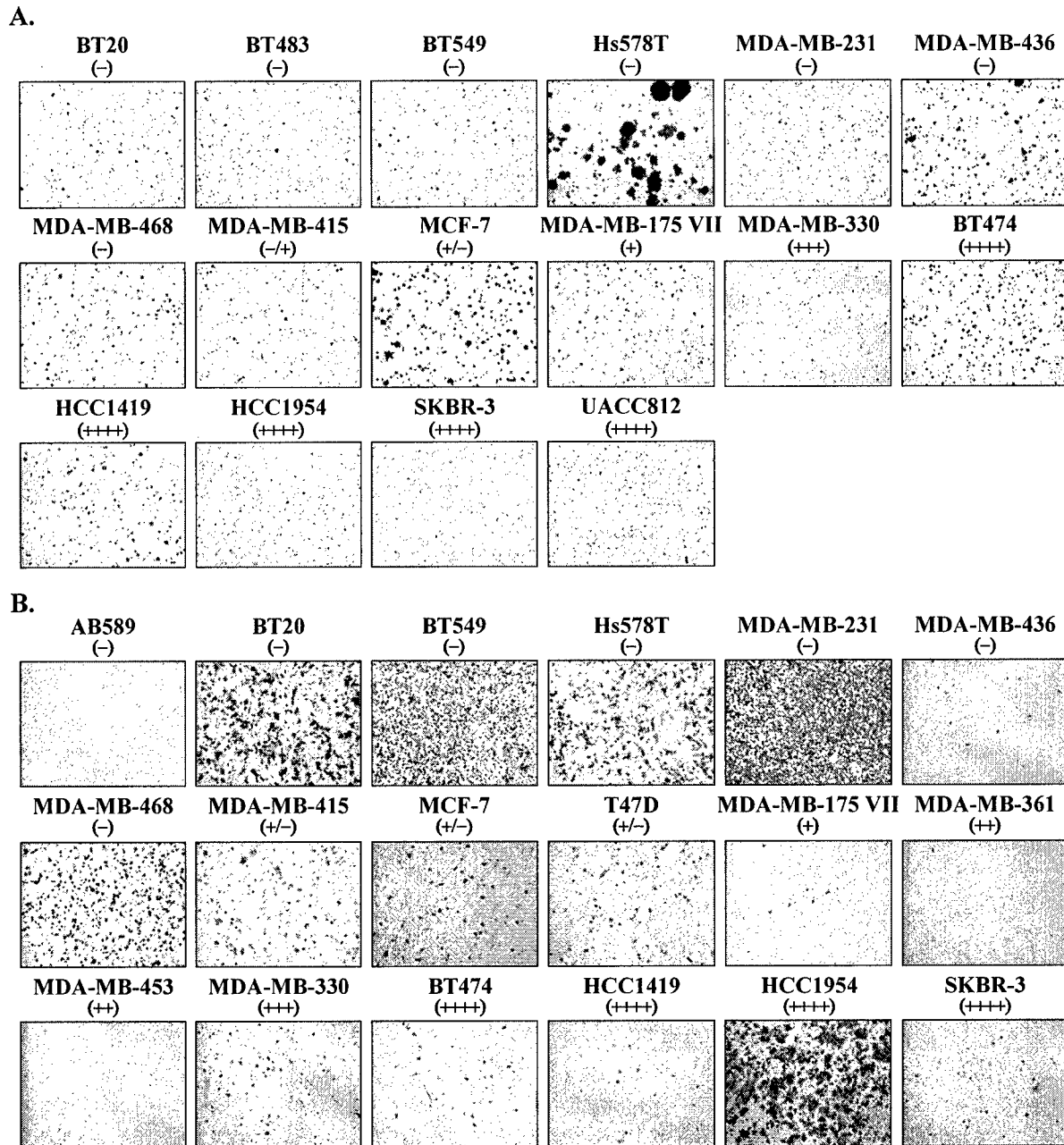


Fig. 2. Colony forming and invasive ability of various breast cancer cell lines. **A.** 1×10^5 cells from various breast cancer cell lines were used in a soft agar colony assay as indicated in the materials and methods. Cultures were replenished with normal growth media every 3 days. Photomicrographs were taken 18 days after initiation of the assay. **B.** Normal and various breast cancer cell lines were used in a 18hr in-vitro invasion assay using Boyden chambers as described in the materials and methods. Photomicrographs of the invaded cells were taken at 40X magnification. The “+” and “-” signs denote the relative levels of ErbB2 expression.

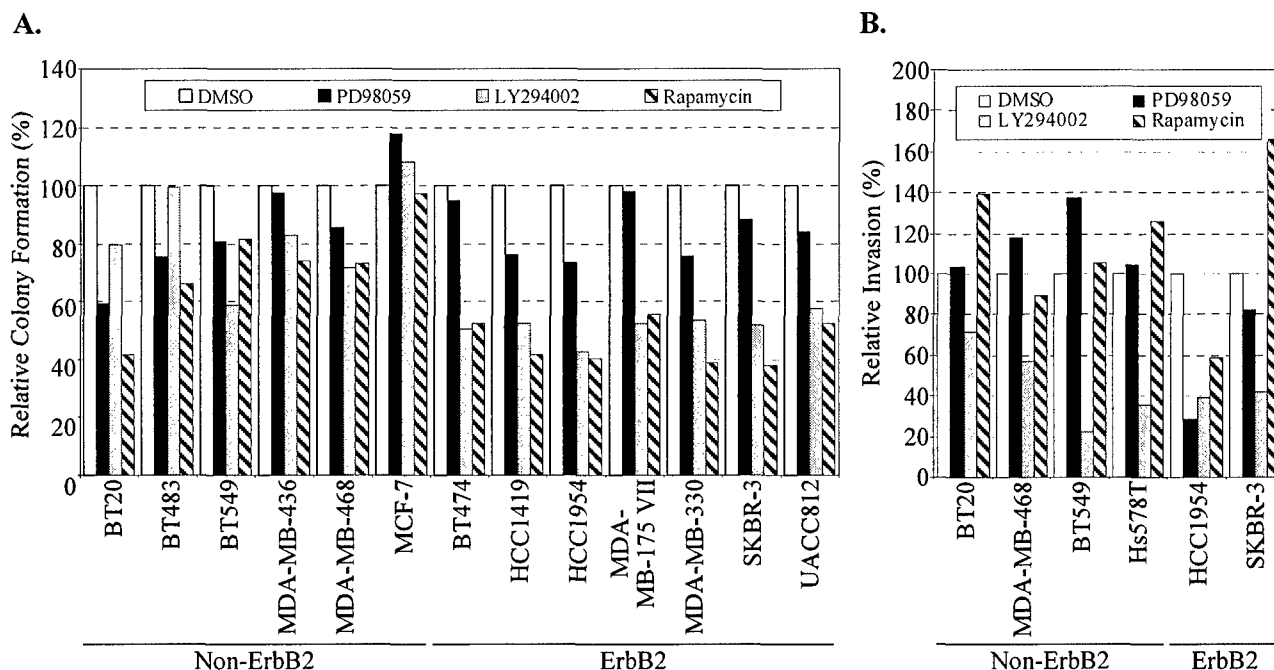


Fig. 3. Pharmacological inhibition of colony forming and invasive ability in various breast cancer cells. **A.** 1×10^5 cells from various breast cancer cell lines were used in a soft agar colony assay in the presence of DMSO, 10 μ M LY294002, 25 μ M PD98059 or 10ng/ml Rapamycin. Cultures were replenished with normal growth media supplemented with the appropriate inhibitor every 3 days. 3 representative fields were photomicrographed 18 days after initiation of the assay. Colony numbers from the 3 fields were quantified using ImageTool 3.0 (UTHSCSA) and normalized against untreated (DMSO) cells. **B.** Invasion assays were performed as above in the presence of DMSO, 10 μ M LY294002, 25 μ M PD98059 or 10ng/ml Rapamycin and 3 representative fields of the invaded cells were photomicrographed. The invasion levels relative to untreated cells (DMSO) were quantified. The results represent the average of 2 independent experiments.

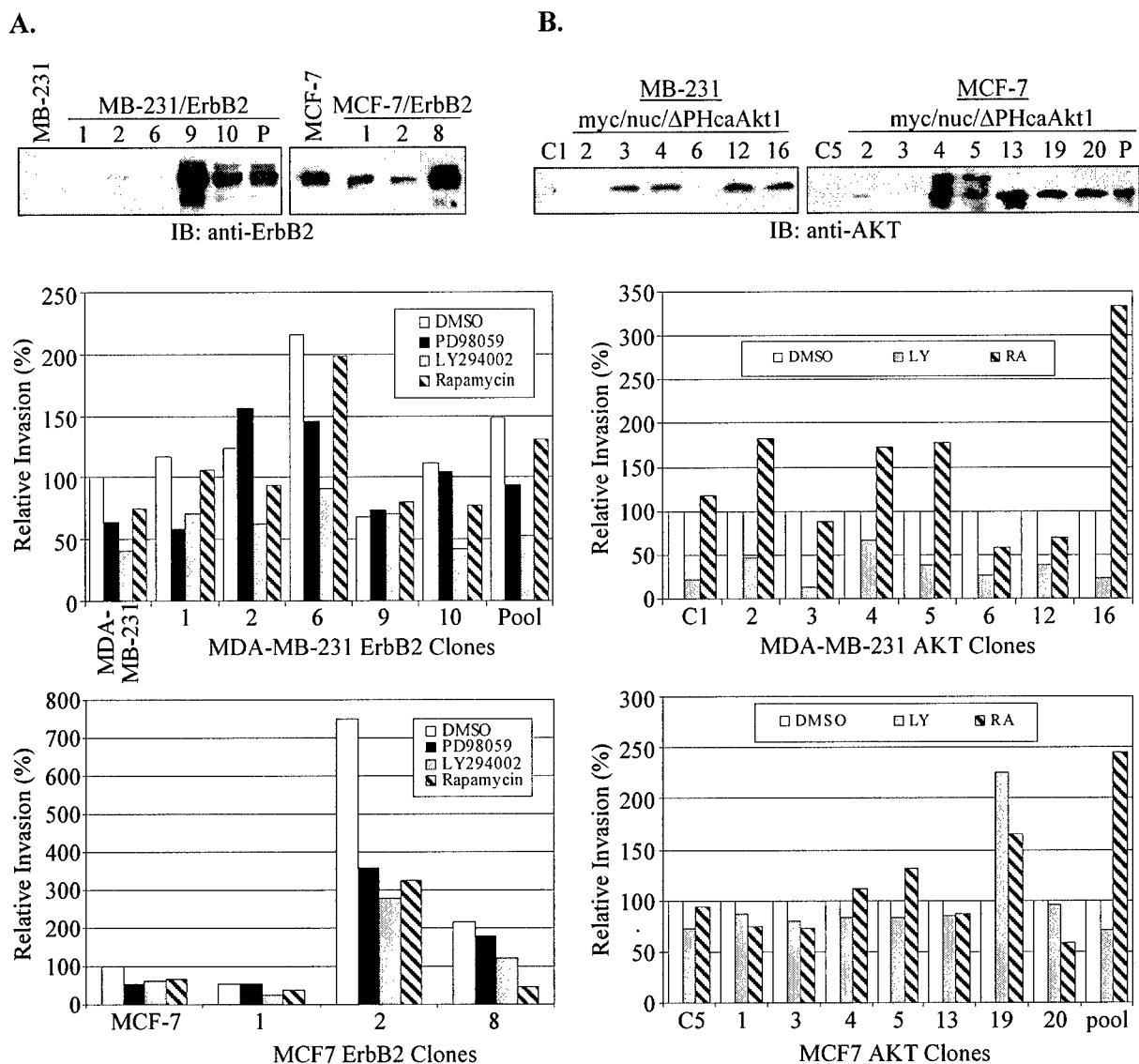


Fig. 4. Effect of forced ErbB2 and AKT1 expression on invasion activity and their sensitivity to drug inhibition. A. ErbB2 and myc/nuc/DPHcaAkt1, an exclusively nuclear targeted constitutively activated AKT mutant, were stably transfected into MCF7 or MDA-MB-231 cells and clones selected. Total cell lysates were prepared from various clones and protein amounts quantified. Equivalent amounts of lysate were separated by SDS-PAGE followed by western blotting with the indicated antibodies to detect exogenous expression of either ErbB2 or myc/nuc/DPHcaAkt1. Invasion assays were performed using the either the (B.) ErbB2 or (C.) myc/nuc/DPHcaAkt1 overexpressing clones in the presence of DMSO or the indicated pharmacological inhibitors and 3 representative fields of the invaded cells were photomicrographed. The invasion levels relative to untreated cells (DMSO) were quantified. Results shown in histograms represent the average of 2 independent experiments

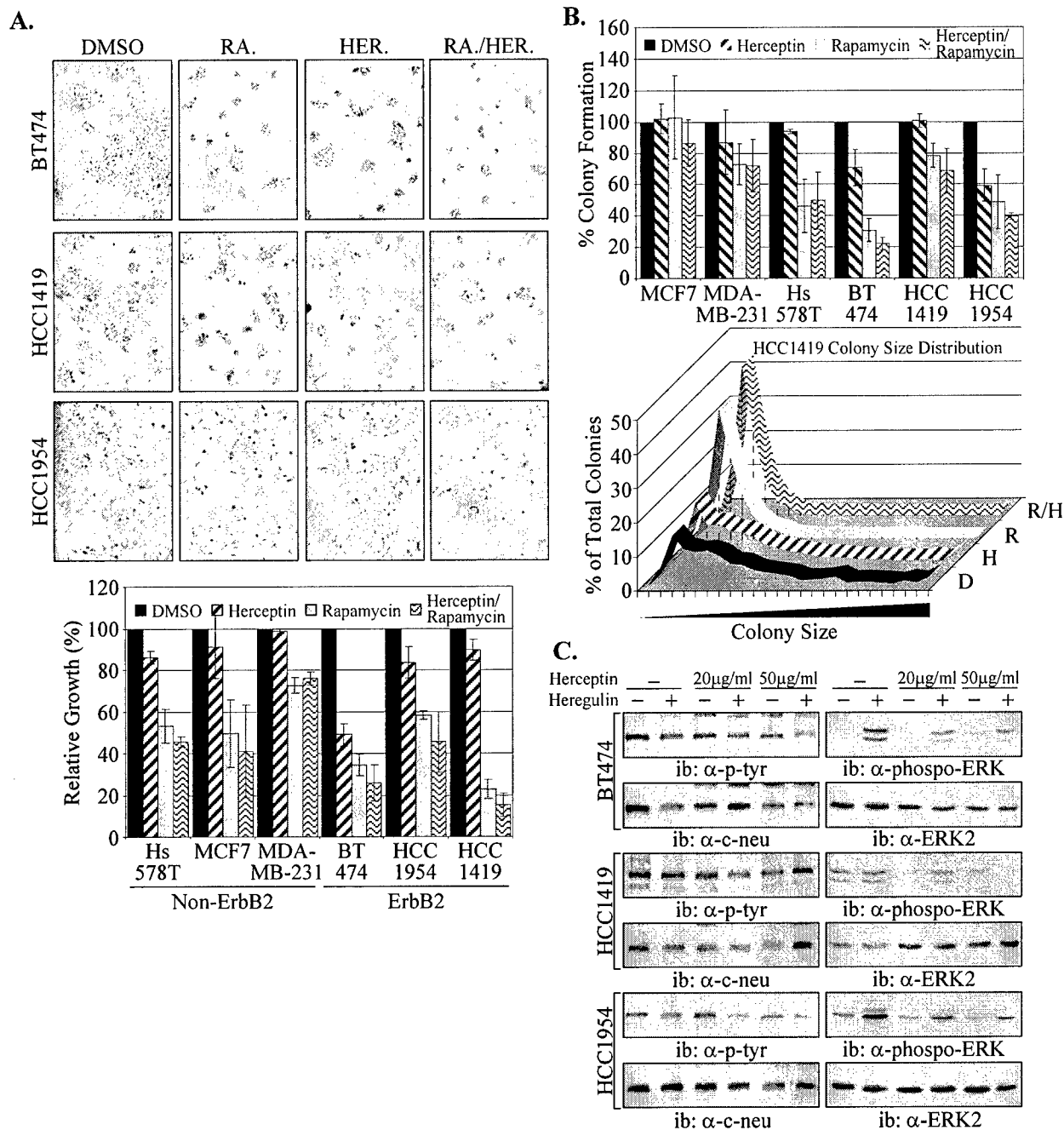


Fig. 5. Effect of Rapamycin and/or Herceptin on colony formation and monolayer growth. A. 2×10^4 cells were plated in duplicate 12-well plates. One day after plating, medium was replaced with fresh medium containing DMSO, 5ng/ml rapamycin, 50 μ g/ml herceptin or a combination of rapamycin and herceptin. Cells were photographed (A. top) and counted 72 hours after treatment (A. bottom) The result represent 3 independent experiments. B. 2×10^4 of the indicated breast cancer cells were used for a soft agar colony assay in a 12-well plate under similar conditions as above. DMSO, 5ng/ml rapamycin, 50 μ g/ml herceptin or a combination of rapamycin and herceptin was included in the top agar at the beginning of the assay as well as the overlaying media that were subsequently added every 3 days. Triplicate

digital photomicrographs were taken 14 days after initiation of the assay. The numbers of colonies were quantified. Colony numbers were normalized to the uninhibited (DMSO) control. The colony size distribution of HCC1419 is also shown (B. bottom panel). Histograms represent composite results from 3 independent experiments. C. BT474, HCC1419 and HCC1954 breast cancer cells were starved and left untreated or treated with the indicated concentrations of herceptin for 18 hrs. The cells were then left as is or stimulated with heregulin for 10 minutes before preparation of total cell lysates. 20 μ g of total cell lysate was separated by SDS-PAGE followed by western blotting with the indicated antibodies. Protein was detected via Western-STAR detection reagent (Perkin Elmer).

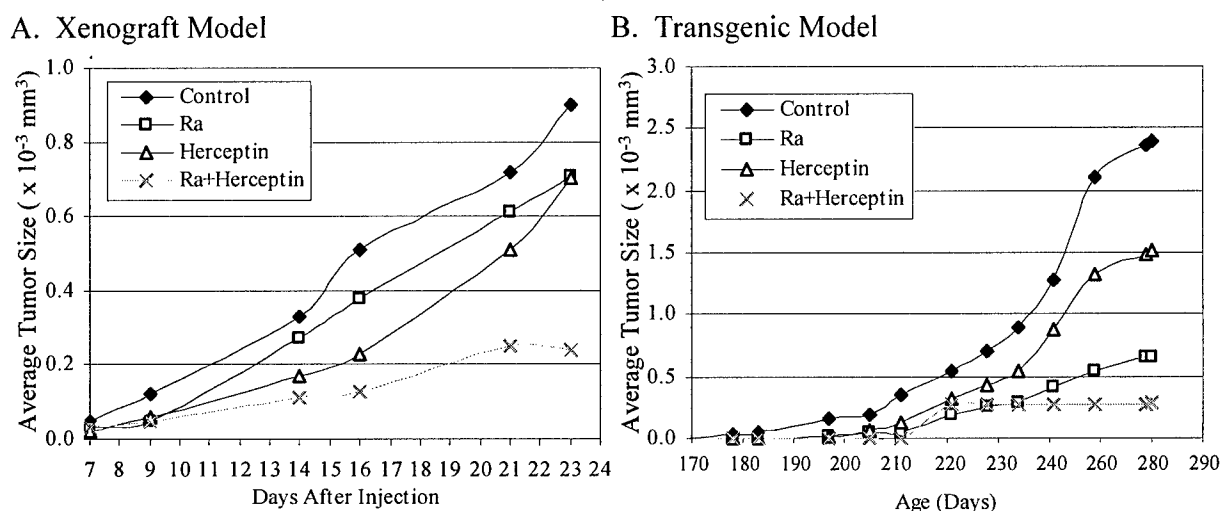


Fig. 6. Effect of rapamycin and herceptin on tumorigenicity. A. Xenograft model. 2 X 10⁶ MCF-7 14 cell in 0.1ml PBS were injected into each nude mouse at the flank subcutaneously. One week post-injection, when the tumor nodule appeared, drug administration was started at the site of tumor cell injection twice a week. The 4 treatment groups, with 5 mice each, are PBS, rapamycin at 10ng/ml in PBS, herceptin at 50 μ g/ml in PBS and a combination of both. Tumor size was measured using a caliper twice a week. **B. Transgenic model.** Female FVB/N-TGN mice harboring ErbB2 under a MMTV LTR control were maintained until 140 days old. At that time they were divided into 4 groups with 10 mice each. The 4 treatment groups are 1. Control vehicle solvent (PBS containing 5% PEG400, 4% ethanol and 5% Tween 80 (PET)), 2. rapamycin at 4mg/kg in 0.1ml PET vehicle solvent, 3. herceptin at 4mg/kg in 0.1 ml PBS and 4. a combination of both. All drugs were prepared in 0.1 ml PBS or vehicle solvent and administered intraperitoneally twice a week. Tumor size was measured using a caliper twice a week.

Effect of Rapamycin and Herceptin on Tumor Growth at End Point					
Treatment	Tumor Incidence	Total Tumor Weight (gms)	Average Tumor Weight (gms)	% Mice with Multiple Tumors	Average # of Tumor/Mice
Control	58% (7/13)	15.8	1.21	46% (6/13)	1.3
Rapamycin	50% (5/10)	3.4	0.34	20% (2/10)	0.8
Herceptin	44% (4/9)	6.8	0.76	33% (3/9)	0.9
Rapamycin/ Herceptin	40% (4/10)	1.9	0.19	0% (0/10)	0.4

Table 1. Effect of Rapamycin and Herceptin on Tumor Growth at End Point. At the endpoint (260 days old), the transgenic mice were euthanized and dissected. The incidence of tumors, multiple tumors and the total tumor weights were noted for each mice in each group.

II. Selection and characterization of highly invasive breast cancer cells

To explore molecular basis for cell migration/invasion activity, we have adopted the in vitro Boyden chamber invasion assay to select for highly invasive cells. The isolation and initial characterization of the highly invasive lines derived from the normal breast epithelial line AB589 and two breast cancer lines, MCF7 and MBA-MD-453 are described here. These selected invasive lines have been called AB589I4, MCF7I4 and MDA-MB-453I4 respectively. All the selected cells assume a fibroblast-like morphology and have greatly increased invasive capability (Fig. 7A,B). In addition to the selected invasive ability, the selected cells also had attained a dramatically increased growth rate and enhanced colony forming ability (Fig. 7C,D). The colony forming ability of AB589I4 and MB453I4, but less so for MCF7I4, was very sensitive to inhibition by LY294002 and rapamycin (Fig. 8A). By contrast, the colony forming ability of all of them was relatively resistant to the inhibition of PD98059. Although LY294002 and PD98059 did not reduce the colony number of MCF7I4, they resulted in much smaller colonies as shown by the size distribution of the colonies. The reason for the increased colony formation of MCF7I4 treated with rapamycin is currently unknown and is to be further examined. The growth rate of the selected cells was surprisingly resistant to the inhibition of all three drugs tested (Fig. 8C). The invasion activity of the selected cells was sensitive to the inhibition of LY294002 and rapamycin especially that of the AB589I4 cells (Fig. 8B). However, rapamycin reproducibly enhanced the invasion activity of MB231I4, which also needs to be further scrutinized for the underlying basis. The invasion activity was also inhibited by PD98059 in all three lines. When inoculated into athymic mice, the MCF7I4 and MB453I4 cells showed a much higher tumorigenicity than the respective parental lines, which had no significant tumorigenicity under such condition (Fig.9). Potential metastasis of the cells to various distant organs is currently being investigated.

What is the origin of the selected highly invasive cells? It is possible that upon repeated passages of the original line, some spontaneously variant cells with increased migration/invasion activity have emerged and co-exist with the parental cells, although in a tiny proportion. Those cells were then selected out by the invasive/migration screening assay. Based on this hypothesis, the variant cells were unable to outgrow the parental cells until they are selected out and separated from the parental cells since they have never become apparent prior to the selection despite repeated passages of the parental lines. To test this hypothesis, reconstitution experiments were performed. The cells of each parental line was mixed with the corresponding selected cells at a ratio of 200 to 1 and then transferred regularly upon confluence. The selected cells in all three lines became apparent in 3 to 5 generations of transfer and eventually outgrew the parental cells (data not shown). The observation does not support the possibility of pre-existed minor variants. The selected high invasive cells are genetically stable since they retain the ability upon subsequent repeated passages for more than 10 generations. Therefore, they are likely to represent the newly arisen variants during the protocol of selection.

To begin to explore the molecular basis underlying the enhanced invasion activity of the selected cells, the activity of various signaling pathways in each pair of parental and selected lines was compared. The cell-matrix- and cell-cell interaction-mediated signaling molecules were analyzed. The selected invasive cells have higher level of fibronectin, β 1-integrin, Crk and phosphorylated paxillin (Fig.10B). In addition, all the selected cells contain a slower migrating species of the p120^{ctn} replacing the faster migrating counterpart protein present in the parental cells suggesting that p120^{ctn} is hyperphosphorylated (Fig.10B). The p120^{ctn} is associated with the cytoplasmic domain of E-cadherin and plays a role in cell-cell interaction and

cadherin/catenin-mediated signaling. Upon serum stimulation, the selected cells gave rise to more tyrosine phosphorylated cellular proteins especially in the molecular weight range of 50 to 80 kD (Fig.10A). The MAK kinase, especially the slower migrating species, was activated to a much higher degree in response to serum stimulation in the selected cells (Fig.10A). However, activation of Akt was similar in parental and selected cells. These observations strongly suggest that the selected cells have the potential to lunge a significantly enhanced signaling functions resulting from cell-cell, cell-matrix interaction, as well as from growth factor stimulation. Further analysis is needed to dissect the specific signaling pathways involved in promoting invasion versus those for growth and anoikis resistance. Preliminary results from microarray analysis of the gene expression profiles in the parental and selected cells revealed that several cell cycle regulatory proteins were significantly altered (data not shown). It is note worthy that cyclin D1 level was greatly increased in the selected cells while opposite was true for cyclin A (Fig.10C). Whereas no significant difference was detected for CDK2 and CDK4, CDK6 was greatly increased in MCF14 and MB45314 cells (Fig.10C). The changes in those cell cycle regulatory proteins could in part account for the increased growth rate of the selected cells.

III. Transient tumor organ culture and tumor cell culture, biochemical analysis and drug sensitivity test

Due to the difficulty in procuring sizable breast tumor specimens for the intended analysis, we have chosen instead the more readily available serous papillary ovarian tumor, which is closely resembles that of the breast tumor in their embryonic origin of the tumor cells. Some examples of the tumor organ cultures prepared as described in Method are shown in Fig. 11. The cultures usually last for about 3 to 4 weeks with regular medium replenishing. The growth around the edge of the specimen was noticed and the cells usually spread along the matrix support. The cultures were tested for inhibition by rapamycin and/or carboplatin, the currently most frequently used chemotherapeutic drug for ovarian cancer. Markedly inhibition by rapamycin alone and, in particular, in combination with carboplatin was noticed as judged by the growth pattern or by ³H-thymidine incorporation (Fig. 12). Examples of the fresh tumor-derived monolayer cultures are shown in Fig. 13A. They showed rather variable invasion activity (Fig. 13B). The inhibition of monolayer growth and invasion activity of cells derived from a serous papillary tumor and the counterpart normal fallopian epithelium by rapamycin and carboplatin is shown in Fig. 14. Rapamycin and carboplatin showed enhanced inhibition on growth and invasion activity. The normal cells were relatively resistant to the inhibition for growth under such condition. Analyses of several normal and tumor sepecimens for expression and/or activation of a number of receptor protein tyrosine kinases (RPTKs) and their down stream signaling molecules are shown in Fig. 15. The tumor specimens showed distinct expression profile of RPTKs, MAP kinase, Akt and cell-cell interacting signaling molecules, E-cadherin and catenins. Most of the tumor cells have a higher level of ErbB2 and IGF-1 receptor. These observations suggest a synergistic inhibitory effect of rapamycin and carboplatin on the growth and invasion activity of ovarian tumors.

F. Publication:

1. Results described in I have been presented in the "2005 Era of Hope Symposium" in a podium and a poster presentation. A manuscript is prepared for submission to Cancer Research.
2. The results described in II and III are also being written up for submission for publication.

Figures

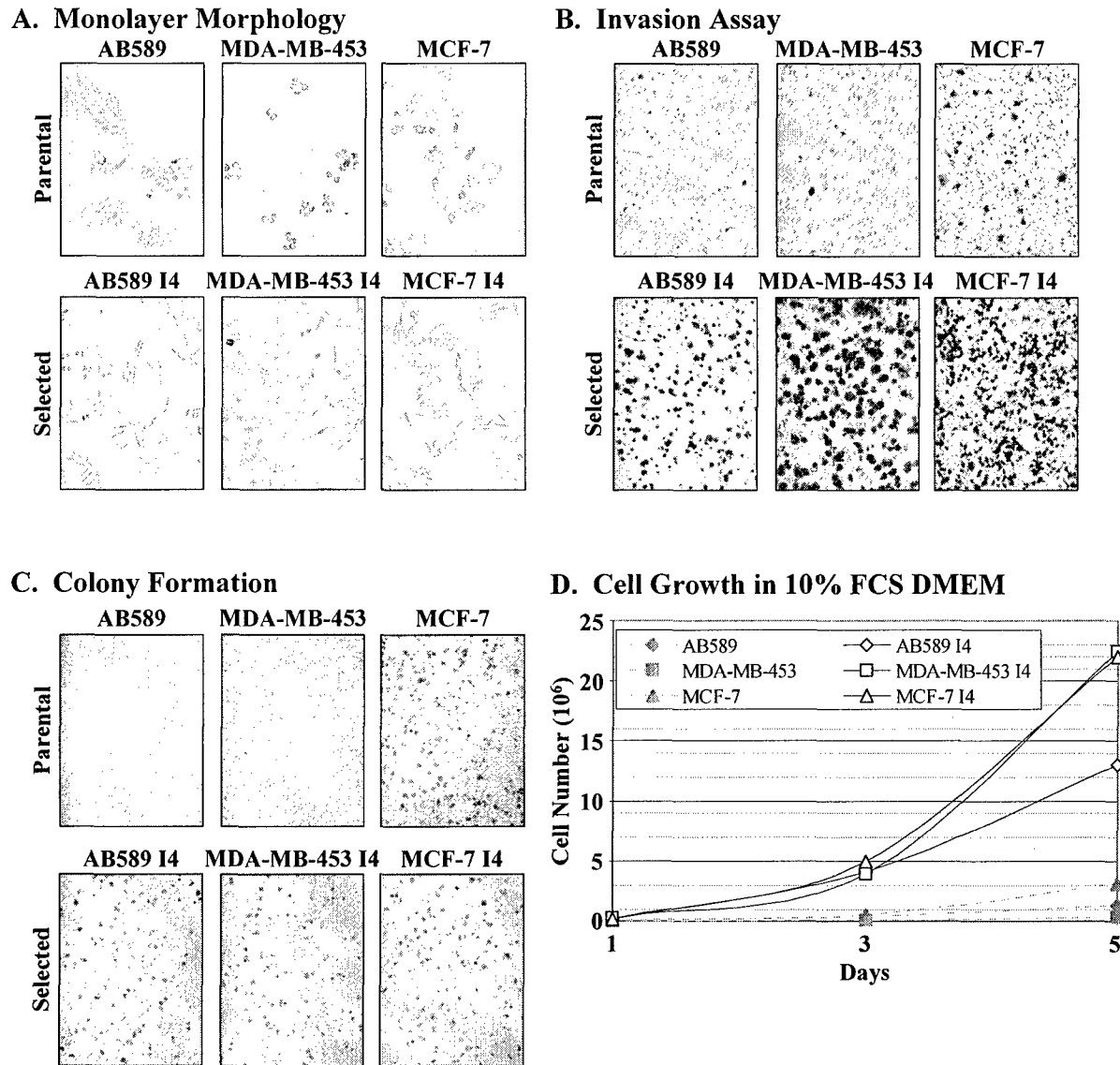
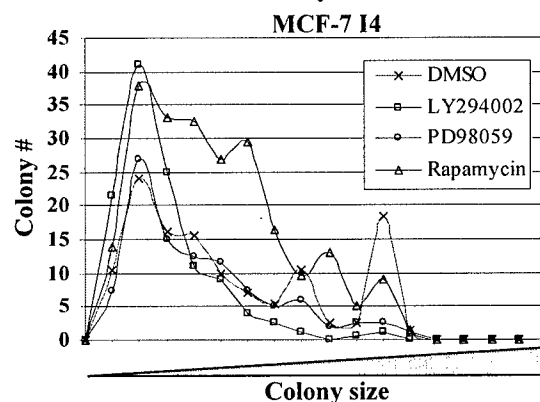
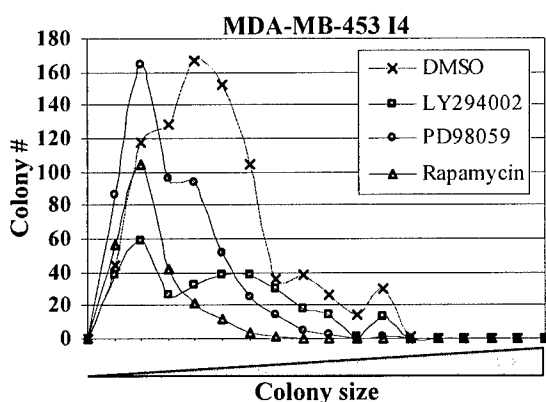
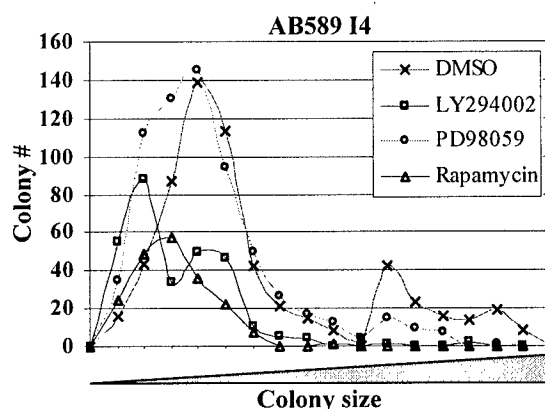


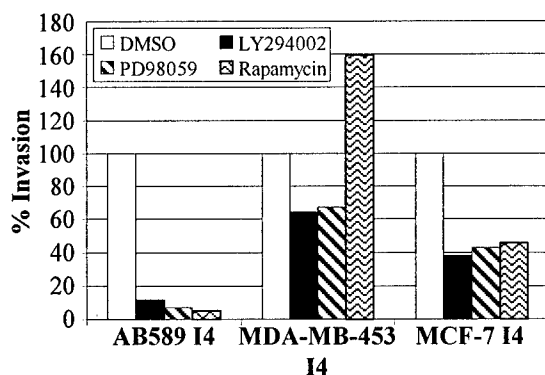
Fig. 7. Selection and characterization highly invasive breast cancer cells. 4 rounds of in-vitro invasion selection were performed using Boyden chambers. **A.** The parental and selected cells were plated at a low density and the monolayer morphology photographed at 40X magnification. **B.** The invasive potential of the selected cells (named as I4) were compared to the parentals. The duration of invasion for the AB589, MDA-MB-453, and MCF-7 pairs (parental and selected) were 8, 6 and 12 hrs respectively. **C.** Colony assays were performed using 1×10^5 cells. Cells were fed with 500 μ l of 10% FCS DMEM every 2 days. Photomicrographs were taken at 14 days. **D.** Parallel dishes containing 1×10^4 cells were setup and cell numbers determined on the indicated days

A. Inhibition of Colony Formation

	AB589 I4	MDA-MB-453 I4	MCF-7 I4
DMSO	611	864	124
LY294002	297	310	117
PD98059	659	540	100
RAPAMYCIN	196	242	229



B. Inhibition of Invasion



C. Inhibition of Growth

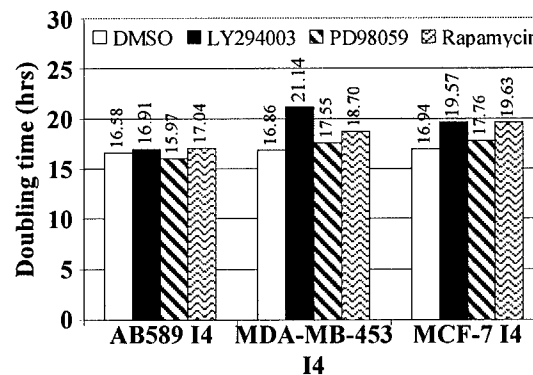


Fig. 8. Inhibition of colony forming, invasion and growth activity of the selected breast cancer cells by different drugs. A. Colony assays were performed in the presence or absence of the indicated inhibitors at the previously stated concentrations. For MCF-7 I4, only 1×10^4 cells were used. After 14 days the colonies were assessed for their numbers and sizes. Upper left panel is a table of total colony numbers. Other panels are graphical representations of the colony size distribution. B. Invasion assay were performed in the presence or absence of the indicated inhibitor as above. Number of invaded cells were determined and the percent invasion relative to untreated (DMSO) cells calculated. C. Cells were grown in 10% FCS DMEM in the absence or presence of the indicated inhibitors. After 48 hrs cell numbers were quantified and compared to initial cell numbers before treatment to calculate doubling times

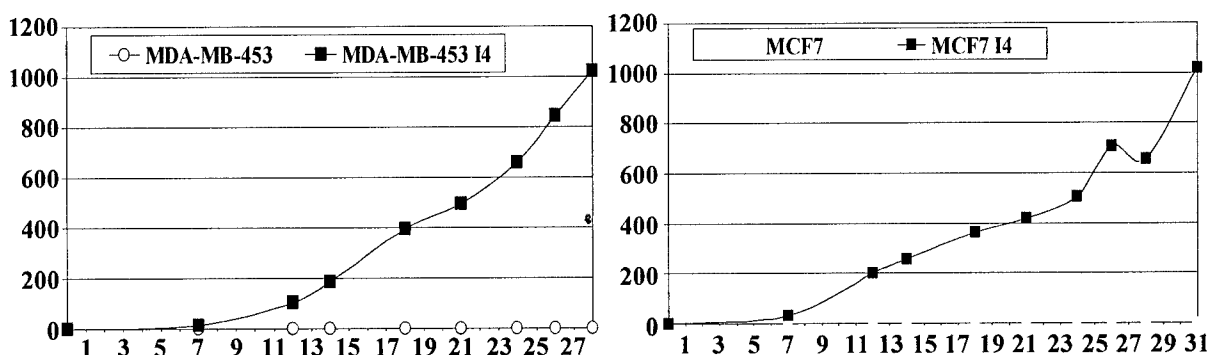


Fig. 9. Tumorigenicity of the MCF7I4 and MDA-MB-453I4 cells. Four groups of nude mice (5 per group) were injected with 1×10^6 cells of MCF7, MCF7I4, MDA-MB-453 or MDA-MB-453I4 in 0.1 ml PBS at flank. The growth of tumor was monitored. Tumor size in mm^3 was measured with a vernier caliper.

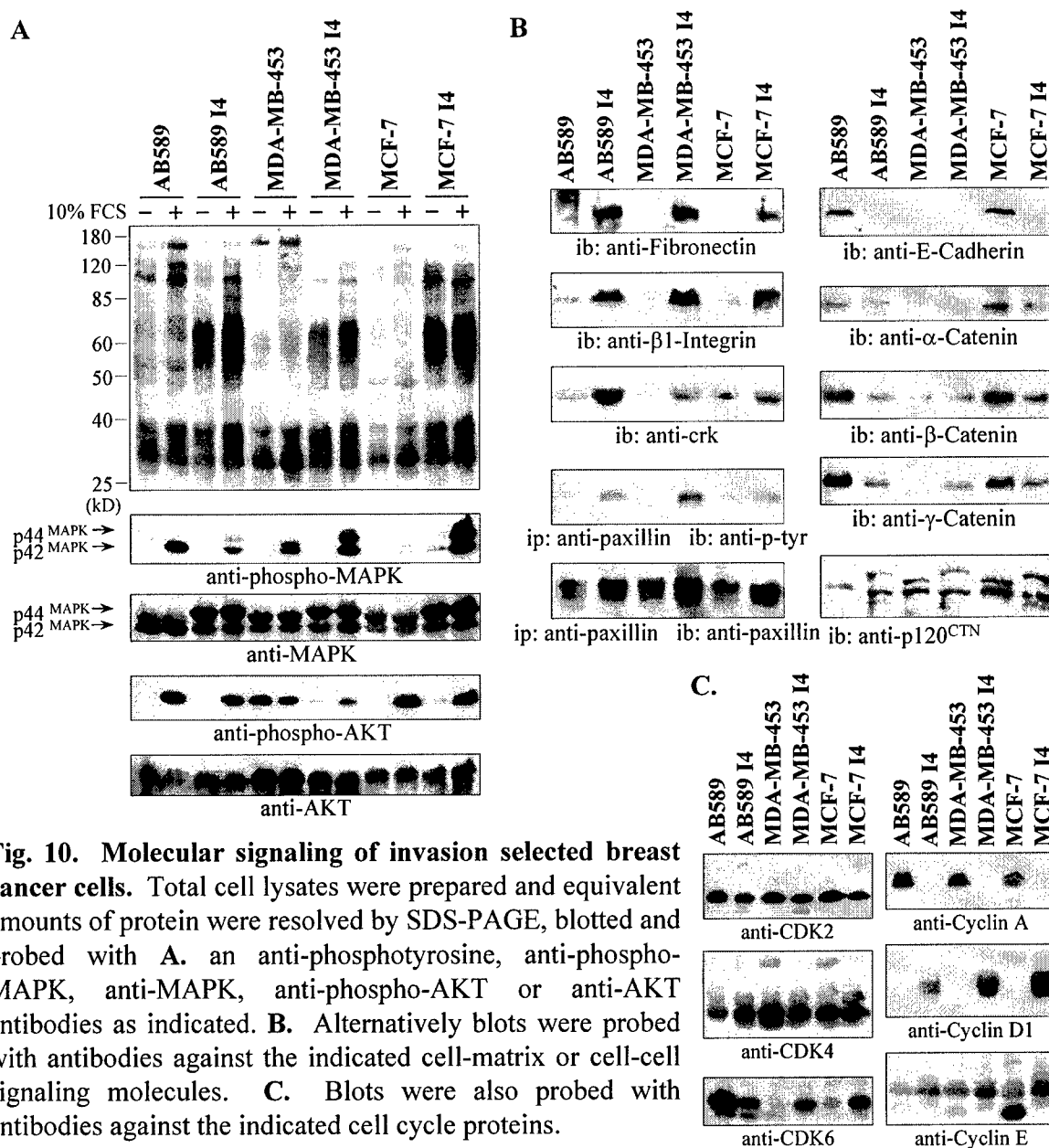


Fig. 10. Molecular signaling of invasion selected breast cancer cells. Total cell lysates were prepared and equivalent amounts of protein were resolved by SDS-PAGE, blotted and probed with **A.** an anti-phosphotyrosine, anti-phospho-MAPK, anti-MAPK, anti-phospho-AKT or anti-AKT antibodies as indicated. **B.** Alternatively blots were probed with antibodies against the indicated cell-matrix or cell-cell signaling molecules. **C.** Blots were also probed with antibodies against the indicated cell cycle proteins.



Fig. 11. Fresh tumors-derived cultures Fresh tumor tissue was cultured on nylon mesh suspended on 5 ml of 10% FCS DMEM. The growth of cells from the primary tissue specimen after one week of cultivation was photographed.

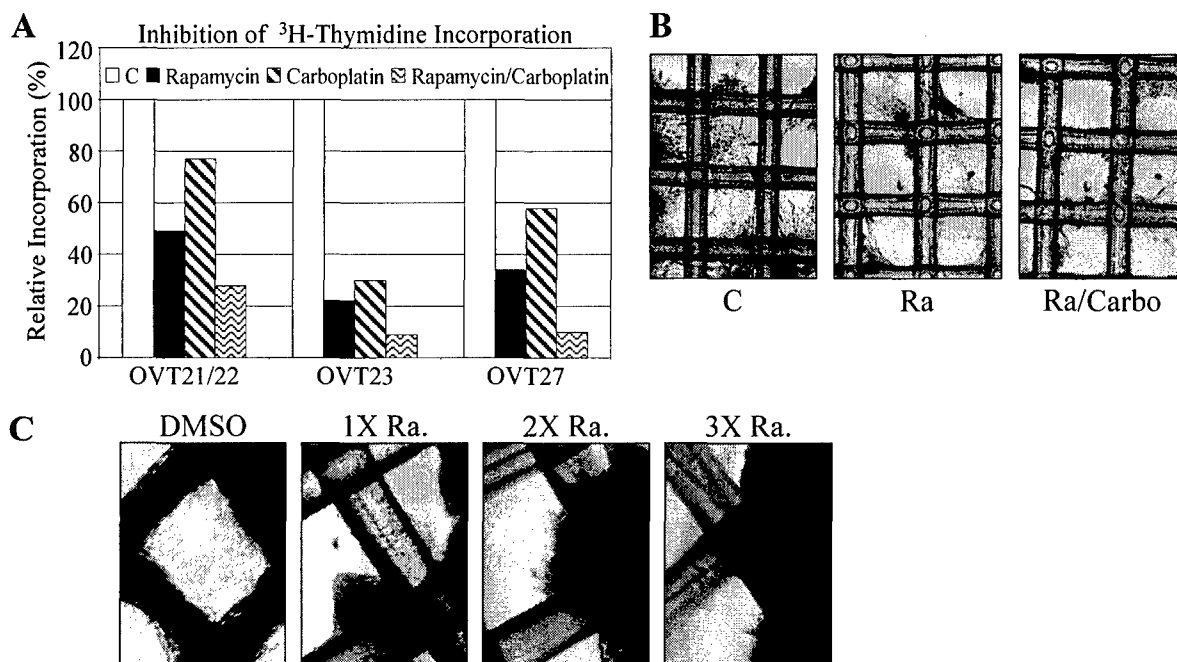


Fig. 12. Effect of Rapamycin and/or Carboplatin on Fresh tumors cultures. A. Inhibition of ^3H -thymidine incorporation in fresh tumors OVT21/22, OVT23 and OVT27 by 10ng/ml rapamycin, 2 $\mu\text{g}/\text{ml}$ carboplatin or both. Histogram represents the average of duplicate organ culture for each tumor. B. Inhibition of OVT22 tumor cell growth by 10ng/ml Rapamycin or 10ng/ml Rapamycin/2 $\mu\text{g}/\text{ml}$ Carboplatin. C. Inhibition of the OVT16 tumor cell growth at 10 (1X), 20 (2X) and 30 (3X) ng/ml of rapamycin.

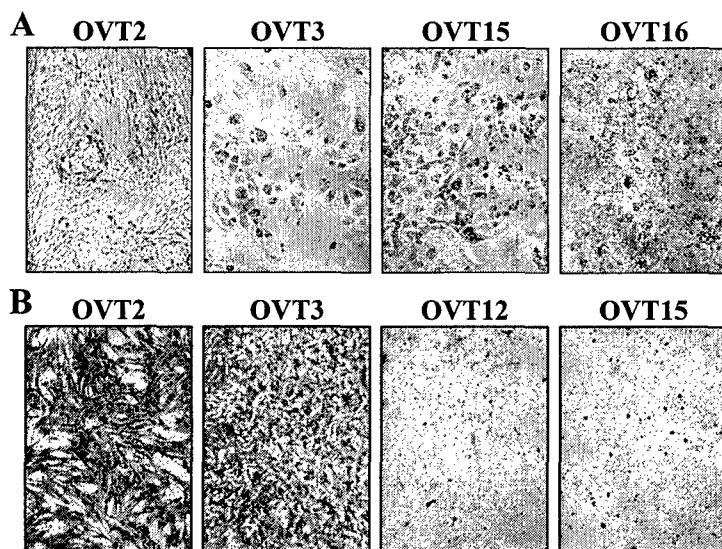


Fig. 13. Fresh tumors-derived cell cultures and the invasion activity of the tumor cells. A. Primary tumor derived cells were cultured in a monolayer. B. Invasion activity of cultured tumor cells 1×10^5 cells of each were assayed for invasion across the Matrigel coated membrane of Boyden chamber for 24 hours.

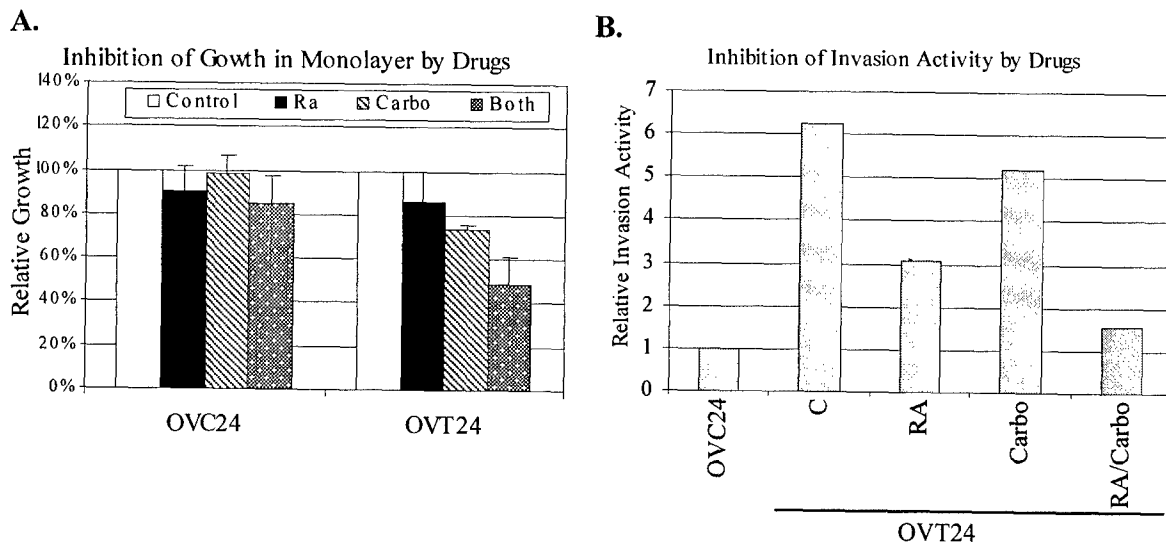


Fig. 14. Inhibition of growth and invasion of fresh tumor-derived cells. The monolayer cultures were prepared from a serous papillary ovarian tumor specimen and the counterpart normal cells from fallopian tube. They were subjected to **A.** growth and **B.** invasion assay. For monolayer growth, 1×10^5 cells were seeded per 6 cm dish in triplicate, the next day medium was changed and replenished with fresh medium with or without the indicated drugs. Cells were counted 3 days alter. For invasion assay, 5×10^4 cells were seeded in the Boyden chamber in duplicate and assayed for invasion for 24 hours as described above in the absence or presence of the indicated drugs. The histograms represent the average values from duplicates of the assay normalized to that of the normal cells.

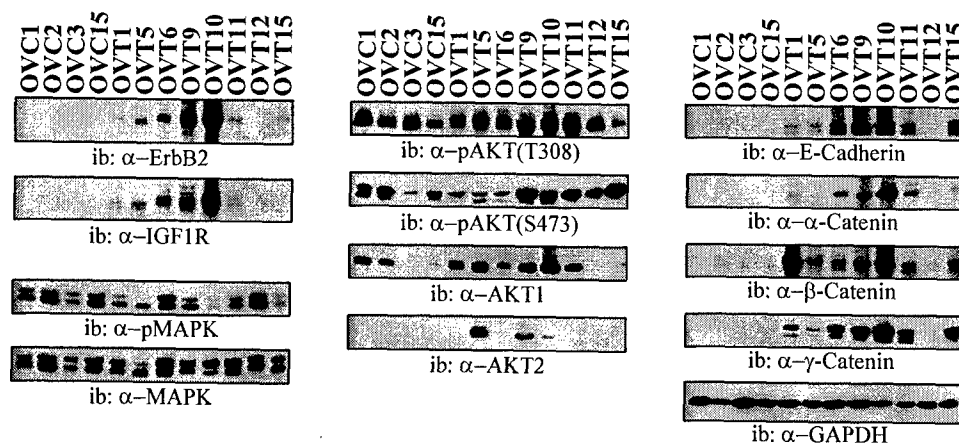


Fig. 15. Analysis of growth factor receptor and cell-cell mediated signaling molecules in various ovarian tumors. Total Cell lysates were prepared from frozen normal tissue (OVC1-C3) and ovarian tumors (OVT1-T15) and 20μg of total cell lysate were resolved by SDS-PAGE and blotted with the indicated antibodies.

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